HUMAN RNase H1 MUTANTS

INTRODUCTION

RNase H hydrolyzes RNA in RNA-DNA hybrids (1). RNase H activity appears to be ubiquitous in eukaryotes and bacteria (2-7). Although RNase H's constitute a family of proteins of varying molecular weight, the nucleolytic activity and substrate requirements appear to be similar for the various isotypes. For example, all RNase H's studied to date function as endonucleases exhibiting limited sequence specificity and requiring divalent cations (e.g., Mg^{2+}, Mn^{2+}) to produce cleavage products with 5' phosphate and 3' hydroxyl termini (8).

Two classes of RNase H enzymes have been identified in mammalian cells (5, 9-10). These enzymes were shown to differ with respect to co-factor requirements and were shown to be inhibited by sulfhydryl reagents (10-11). Although the biological roles of the mammalian enzymes are not fully understood, it has been suggested that mammalian RNase H1 may be involved in replication and that the RNase H2 enzyme may be involved in transcription (12-13).

Recently, both human RNase H genes have been cloned and expressed (11, 14-15). RNase H1 is a 286 amino acid

protein with a calculated mass of 32 kDa (11). The enzyme is encoded by a single gene that is at least 10 kb in length and is expressed ubiquitously in human cells and tissues. The amino acid sequence of human RNase H1 displays strong homology with RNase H1 from yeast, chicken, *E.coli* and the mouse (11). The human RNase H2 enzyme is a 299 amino acid protein with a calculated mass of 33.4 kDa and has also been shown to be ubiquitously expressed in human cells and tissues (14, H. Wu, unpublished data). Human RNase H2 shares strong amino acid sequence homology with RNase H2 from C. elegans, yeast and E. coli (14).

The properties of the cloned and expressed human RNase H1 have recently been characterized (16). The activity of RNase H1 is Mg^{+2} dependent and inhibited by both Mn^{+2} and the sulfhydryl blocking agent N-ethylmaleimide. Human RNase H1 was also inhibited by increasing ionic strength with optimal activity for both KCl and NaCl observed at 10 - 20 mM. The enzyme exhibited a bell-shaped response to divalent cations and pH, with the optimum conditions for catalysis observed to be 1 mM Mg^{2+} and pH 7 - 8. The protein was shown to be reversibly denatured under the influence of temperature and destabilizing agents such as urea. Renaturation of human

RNase H1 was observed to be highly cooperative and did not require divalent cations. Furthermore, RNase H1 displayed no tendency to form intermolecular disulfides or to form homo-multimers. Human RNase H1 was shown to bind selectively to "A-form" duplexes with 10-20-fold greater affinity than that observed for E. coli RNase H1 (16-17). Finally, human RNase H1 displays a strong positional preference for cleavage, i.e., the enzyme cleaves between 8 and 12 nucleotides from the 5'-RNA-3'-DNA terminus of the duplex.

Many of the properties observed for Human RNase H1 are consistent with the *E. coli* RNase H1 isotype, (e.g., the cofactor requirements, substrate specificity and binding specificity) H1 (16-17). In fact, the carboxy-terminal portion of human RNase H1 is highly conserved with the amino acid sequence of the *E. coli* enzyme, (region III, Figure 1). The glutamic acid and two aspartic acid residues of the catalytic site, as well as the histidine and aspartic acid residues of the proposed second divalent cation binding site of the *E. coli* enzyme are conserved in human RNase H1 (18-21). In addition, the lysine residues within the highly basic α-helical substrate-binding region of *E. coli* RNase H1 are also conserved in the human enzyme.

Despite these similarities, the structures of the two enzymes differ in several important ways. For example, the amino acid sequence of the human enzyme is approximately 2-fold larger than the *E. coli* enzyme. The additional amino acid sequence of the human enzyme extends from the amino-terminus of the conserved *E. coli* RNase H1 region and contains a 73 amino acid region homologous with a double-strand RNA (dsRNA) binding motif, (region I, Figure 1). The conserved *E. coli* RNase H1 region at the carboxy-terminus is separated from the dsRNA-binding domain of the human enzyme, by a 62 amino acid region, (region II, Figure 1). Although the role of both regions I and II remain unclear, the dsRNA-binding domain of human RNase H1may account for the observed positional preference for cleavage displayed by the enzyme as well as the enhanced binding affinity of the enzyme for various polynucleotides (16).

In this study we have explored the roles of the conserved amino acids of the catalytic site and the basic substrate-binding domain (region III), the roles of the dsRNA-binding domain (region I) and the 62 amino acid center region of human RNase H1 (region II), (Fig. 1). We have performed site-directed mutagenesis on the three conserved amino acids of the proposed catalytic site of

human RNase H1 ([D145N], [E186Q], and [D210N]). In addition, the net positive charge of the basic substrate-binding domain was progressively reduced through alanine substitution of two (RNase H1[K226,227A]) and four (RNase H1 [K226,227,231,236A]) of the lysines within this region. Deletion mutants were also prepared in which either the dsRNA-binding domain of region I (RNase H1[ΔI]), or the central region II (RNase H1[ΔII]) was deleted. Finally, a mutant protein representing the conserved *E. coli* RNase H1 region was prepared by deleting both region I and II, (RNase H1[ΔI-II]).

SUMMARY OF INVENTION

The carboxy-terminus of human RNase H1 is highly conserved with *E. coli* RNase H1 and contains the amino acid residues of the putative catalytic site and basic substrate-binding domain of the *E. coli* RNase enzyme. The amino-terminus of human RNase H1 contains a structure consistent with a double-strand RNA (dsRNA) binding motif that is separated from the conserved *E. coli* RNase H1 region of the carboxy-terminus by a 62 amino acid sequence. We have performed site-directed mutagenesis on human RNase H1. These studies showed that although the conserved amino acid residues of the putative catalytic

site and basic substrate-binding domain are required for RNase H activity, deletion of either the catalytic site or the basic substrate-binding domain did not ablate binding to the heteroduplex sustrate. Deletion of the region between the dsRNA-binding domain and the conserved *E. coli* RNase H1 domain resulted in a significant loss in the RNase H activity. Furthermore, this deletion mutant competitively inhibited the cleavage activity of the wild-type enzyme suggesting that this central 62 amino acid region does not contribute significantly to the binding affinity of the enzyme for the substrate. The dsRNA-binding domain was not required for RNase H activity, as the dsRNA-deletion mutants exhibited cleavage rates comparable to the rate observed for wild-type enzyme. Comparison of the dissociation constant of human RNase H1 and the RNase H1[ΔI-II] mutant for the heteroduplex substrate suggested that the greatest contribution to binding is from the region situated within the conserved *E. coli* RNase H1 region of human RNase H1. Finally, comparison of the cleavage patterns exhibited by the mutant proteins with the cleavage pattern for the wild-type enzyme indicates that the dsRNA-binding domain is responsible for the observed strong positional preference for cleavage exhibited by human RNase H1.

FIGURE LEGENDS

Figure 1. Schematic showing the structure of the human RNase H1 mutant proteins. (A) Position of amino acid substitution mutants. Mutants include: aspargine substitution of aspartic acid at position 145 [D145 N], glutamine substitution of glutamic acid at position 186 [E186Q], aspargine substitution of Aspartic acid at position 210 [D210 N], alanine substitution of lysine at positions 226 and 227 [K226,227A] and alanine substitution of lysine at positions 226, 227, 231 and 236 [K226,227,231,236A]. The amino acids of regions I, II and II are represented by, respectively, in bold, underlined and plain lettering. Designations within parentheses indicate amino acid positions of *E. coli* RNase H1. (B) Description of deletion mutants of human RNase H1. RNase H1[ΔI] corresponds to the deletion of region I (amino acid positions 1 - 73), RNase H1[ΔII] corresponds to the deletion of region II (amino acid positions 74 - 135) and RNase H1[ΔI-II] corresponds to the deletion of regions I and II (amino acid positions 1 - 135).

Figure 2. SDS-polyacrylamide gel analysis of wild-type and mutant human RNase H1 proteins. The purified proteins were separated on a 4 - 20% gradient Tris-Glycine gel. The

human RNase H1 mutants are described in figure 1. The human RNase H1 proteins (lanes 1-9) are as follows: wild-type, [D145N], [E186Q], [D210N], [K226,227A], [K226,227,231,236A], [ΔII], [ΔI] and [ΔI-II].

Figure 3. RNase H Cleavage site for wild-type and mutant proteins on the 17-mer RNA-DNA heteroduplex. Digestion of the heteroduplex was preformed as described in Materials and Methods. The RNA sequence (5' → 3') is shown above the DNA sequence. The arrows indicate the sites of enzymatic digestion, and the size of the arrows reflect the relative cleavage intensities. (A) Cleavage pattern for wild-type RNase H1, RNase H1[ΔII] and RNase H1[K226,227A] proteins. (B) Cleavage pattern for RNase H1[ΔI] and [ΔI-II] mutants.

Figure 4. Competitive inhibition of wild-type human RNase H1 activity by mutant proteins. Competition experiments were performed as described in Materials and Methods. The heteroduplex substrate was incubated with the mutant proteins prior to adding the wild-type RNase H1 enzyme. The concentration of the wild-type human RNase H1 enzyme was in excess of the substrate concentration. The concentration of the mutant proteins was 10-fold in excess of the wild-type enzyme. The initial rate for the wild-

type human RNase H1 enzyme alone and in the presence of the RNase H1[D145N], [ΔII] and [K226,227,231,236A] (4xK→A) mutants was determined as described in Materials and Methods.

MATERIALS AND METHODS

Construction of mutant proteins

The mutagenesis of human RNase H1 was preformed using a PCR-based technique derived from Landt, et al. (22). Briefly, two separate PCR were performed using a set of site-directed mutagenic primers and two vector-specific primers (11). Approximately 1 μg of human RNase H1 cDNA was used as the template for the first round of amplification of both the amino- and carboxy-terminal portions of the cDNA corresponding to the mutant site. The fragments were purified by agarose gel extraction (Qiagen, Germany). PCR was preformed in two rounds consisting of, respectively, 15 and 25 amplification cycles (94° C, 30s; 55° C, 30s; 72° C, 180s). The purified fragments were used as the template for the second round of PCR using the two vector-specific primers. The final PCR product was purified and cloned into the expression vector pET17b (Novagen, WI) as described previously (11). The

incorporation of the desired mutations was confirmed by DNA sequencing.

Protein expression and purification

The plasmid was transfected into *E.coli* BL21(DE3) (Novagen, WI). The bacteria were grown in M9ZB medium (24) at 32°C and harvested at OD_{600} of 0.8. The cells were induced with 0.5 mM IPTG at 32°C for 2 h. The cells are lysed in 8M urea solution and the recombinant protein was partially purified with Ni-NTA agarose (Qiagen, Germany).

The human RNase H1 was purified by C4 reverse phase chromatography (Beckman, System Gold, Fullerton, CA) using a 0% to 80% gradient of acetonitrile in 0.1% trifluoroacetic acid/distilled water (%v/v) over 40 min (25). The recombinant protein was collected, lyophilized and analyzed by 12% SDS-PAGE (24). The purified protein and control samples were re-suspended in 6 M urea solution containing 20 mM Tris-HCl, pH 7.4, 400 mM NaCl, 20% glycerol, 0.2 mM Phenylmethylsulfonyl fluoride (PMSF), 5 mM dithiothreitol (DTT), 10 μg/ml each aprotinin and leupeptin (Sigma, MO). The protein was refolded by dialysis with decreasing urea concentration from 6 M to 0.5 M and DTT concentration from 5 mM to 0.5 mM (25). The refolded protein was concentrated 10-fold using a Centricon apparatus (Amicon, MA).

Synthesis of oligonucleotides

The oligoribonucleotides were synthesized on a PE-ABI 380B synthesizer using 5'-*O*-silyl-2'-*O*-bis(2-acetoxyethoxy)methyl ribonucleoside phosphoramidites and procedures described elsewhere (23). The oligoribonucleotides were purified by reverse-phase HPLC. The DNA oligonucleotides were synthesized on a PE-ABI 380B automated DNA synthesizer and standard phosphoramidite chemistry. The DNA oligonucleotides were purified by precipitation 2 times out of 0.5 M NaCl with 2.5 volumes of ethyl alcohol.

Preparation of ^{32}P labeled substrate

The RNA substrate is 5'-end-labeled with ^{32}P using 20 u of T4 polynucleotide kinase (Promega, WI), 120 pmol (7000 Ci/mmol) [γ-^{32}P]ATP (ICN, CA), 40 pmol RNA, 70 mM tris, pH 7.6, 10 mM $MgCl_2$ and 50 mM DTT. The kinase reaction is incubated at 37° C for 30 min. The labeled oligoribonucleotide was purified by electrophoresis on a 12% denaturing polyacrylamide gel (24). The specific activity of the labeled oligonucleotide is approximately 3000 to 8000 cpm/fmol.

Preparation of the heteroduplex

The heteroduplex substrate was prepared in 100 μL containing 50 nM unlabeled oligoribonucleotide, 10^5 cpm of

^{32}P labeled oligoribonucleotide, 100 nM complementary oligodeoxynucleotide and hybridization buffer [20 mM tris, pH 7.5, 20 mM KCl]. Reactions were heated at 90° C for 5 min, cooled to 37° C and 60 u of Prime RNase Inhibitor (5 Prime → 3 Prime, CO) and $MgCl_2$ at a final concentration of 1mM were added. Hybridization reactions were incubated 2 - 16 h at 37° C and β-mercaptoethanol (BME) was added at final concentration of 20 mM.

Determination of initial rates (V_0)

The heteroduplex substrate was digested with 0.5 ng human RNase H1 at 37° C. A 10 μl aliquot of the cleavage reaction was removed at time points ranging from 2 - 120 min and quenched by adding 5 μL of stop solution (8 M urea and 120 mM EDTA). The aliquots were heated at 90° C for two min, resolved in a 12% denaturing polyacrylamide gel and the substrate and product bands were quantitated on a Molecular Dynamics PhosphorImager. The concentration of the converted product was plotted as a function of time. The initial cleavage rate was obtained from the slope (mole RNA cleaved/min) of the best-fit line for the linear portion of the plot, which comprises, in general < 10% of the total reaction and data from at least five time points.

Competition experiments were performed as described for the determination of initial rates with the exception that 20 nM oligodeoxynucleatide, 10 nM oligoribonucleotide and 2.5 ng of the mutant RNase H1 protein. Reactions were digested with 250 pg of wild-type Human RNase H. The reactions were quenched, analyzed and quantitated as described for the determination of initial rates.

Determination of dissociation constants (K_d)

Binding affinities were determined by inhibition analysis (17). The RNA-DNA heteroduplex was prepared as described above except in a final volume of 60 μL and with the concentration of the heteroduplex ranging from 10 nM to 500 nM. The non-cleavable heteroduplex substrate was prepared in 60 μL of hybridization buffer containing equimolar concentrations of oligodeoxynucleotide and complementary 2'-fluoro modified oligonucleotide in excess of the RNA-DNA hybrid. The DNA-2'-flouro duplex was added to the RNA-DNA duplex and the combined reaction was digested with human RNase H1 as described for the determination of initial rates. The reactions were quenched, analyzed and quantitated as described for the determination of initial rates.

RESULTS

The mutant proteins of human RNase H1 prepared for this study are described in Figure 1. Analysis of human RNase H1 and the mutant proteins by SDS-polyacrylamide gel electrophoresis is shown in Figure 2. As expected, mutant proteins containing amino acid substitutions, (e.g., D145N, E186Q, D210N, K226,227A and K226,227,231,236A) exhibited molecular weights similar to the 32 kDa wild-type enzyme (lanes 1 - 6). The RNase H1[ΔI] mutant in which the dsRNA-binding domain was deleted resulted in a 213 amino acid protein with an approximate molecular weight of 23 kDa (lane 7). The deletion of the 62 amino acid center portion of human RNase H1 (RNase H1[ΔII]) resulted in a 224 amino acid protein with an approximate molecular weight of 25 kDa (lane 8). Finally, the deletion of both the dsRNA-binding domain and the central region of the enzyme (RNase H1[ΔI-II]) resulted in a 151 amino acid protein containing the conserved *E. coli* RNase H1 sequence and with an approximate molecular weight of 17 kDa (lane 9).

The initial cleavage rates (V_0) were determined for the human RNase H1 enzyme and the mutant proteins using a 17 nucleotide long RNA/DNA heteroduplex, (Table 1).

Table I. Initial cleavage rates for wild-type and mutant human RNase H1 proteins. Initial cleavage rates were determined as described in Materials and Methods. The initial cleavage rates are an average of $n \geq 3$ measurements. *Detection limit = cleavage rates resulting in < 1% of the heteroduplex substrate over 60 min.

Human RNase H1 Protein	V_0 (pM min^{-1})*
Wild-type Rnase H1	658±130
RNase H1[D145N]	below detection limit
RNase H1[E186Q]	below detection limit
RNase H1[D210N]	below detection limit
RNase H1K226,227A]	8.1±0.2
RNase H1K226,227,231,236A]	below detection limit
RNase H1 [ΔI]	488±38
RNase H1[ΔII]	11±2
RNase H1[ΔI-II]	610±20

Substitution of any one the three amino acids comprising the proposed catalytic site of human RNase H1, (e.g., [D145N], [E186Q], and [D210N]) ablated the cleavage activity of the enzyme. The RNase H1 [K226,227A] mutant exhibited an initial cleavage rate almost two orders of magnitude slower than the rate observed for the wild-type enzyme. The alanine substitution of two remaining lysine residues within the basic substrate binding domain (RNase H1 [K226,227,231,236A]) resulted in cleavage activity below the detection limit of the assay.

The initial cleavage rate for the RNase H1[ΔI] mutant in which the dsRNA-binding domain was deleted was 30% slower than the initial cleavage rate observed for the wild-type enzyme (Table 1). The deletion of region II of human RNase H1 resulted in an even greater reduction in the cleavage rate. In this case, the initial cleavage rate observed for the wild-type enzyme was approximately 60-fold faster than the rate observed for the RNase H1[ΔII] mutant. Conversely, the initial cleavage rate for the mutant protein in which both regions I and II were deleted (RNase H1[ΔI-II]) was comparable to the initial cleavage rate observed for the wild-type enzyme.

The positions of the cleavage sites for the wild-type and mutants of human RNase H1in the heteroduplex substrate are shown in Figure 3. As previously observed, human RNase H1 exhibited a strong positional preference, i.e., 8 to 12 nucleotides from the 5'-RNA/3'-DNA terminus of the duplex (Fig. 3A). A similar cleavage pattern was observed for both the RNase H1[K226,227A] substitution mutant and the RNase H1[ΔII] deletion mutant. The RNase H1[ΔI] and H1[ΔI-II] deletion mutants exhibited broader cleavage patterns on the heteroduplex substrate, with cleavage sites ranging from 7 to 13 nucleotides from the 5'-terminus of the RNA (Fig. 3B).

Experiments were performed to determine whether the inactive mutants of human RNase H1 competitively inhibit the cleavage activity of the wild-type enzyme. These experiments were performed with the enzyme concentration in excess of the substrate concentration and with the concentration of the mutant protein in excess of the wild-type enzyme concentration. All three of the mutant proteins tested were observed to competitively inhibit the cleavage activity of human RNase H1 (Fig. 4). For example, the initial cleavage rate of human RNase H1 alone was determined to be 6-fold faster than the initial cleavage rate for human RNase H1 in the presence of the RNase H1[D145N] mutant. The initial cleavage rate of human RNase H1 in the presence of the region II deletion mutant (RNase H1[ΔII]) was approximately 50% slower than the rate observed for human RNase H1 alone. Finally, the initial cleavage rate for human RNase H1 in the presence of the RNase H1[K226,227,231,236A] mutant was approximately 60% slower than the rate observed for human RNase H1 alone.

The binding affinities of human RNase H1 and the RNase H1[ΔI-II] mutant were determined indirectly using a competition assay as previously described (17). Briefly, the cleavage rate of the RNA/DNA heteroduplex was determined at a variety of substrate concentrations in

both the presence and absence competing noncleavable DNA/2'F heteroduplex. The dissociation constant (K_d) of human RNase H1 for the DNA/2'F heteroduplex was 75 nM. The RNase H1[ΔI-II] mutant exhibited a K_d of 126 nM for the DNA/2'F heteroduplex (Table II).

Table II. Binding Constants of RNase H1 proteins. K_d measurements were determined as described in Materials and Methods. The K_d value for *E. coli* RNase H1 was derived from previously reported data (17). The dissociation constants for human RNase H1 proteins are derived from ≥ 2 slopes of Lineweaver-Burk analysis.

RNase H1 Protein	**K_d(nM)**
Human RNase H1[ΔI-II]	75±8
Human RNase H1 [ΔI-II]	126±22
E. coli RNase H1	1600

DISCUSSION

Structure of human RNase H1

The human RNase H1 protein can be divided into three regions (Fig. 1). Region I, located at the amino-terminus of the enzyme, contains a structure consistent with a dsRNA-binding motif. Region II consists of a 62 amino acid region between the dsRNA-binding domain and the conserved

E. coli RNase H1 region. Lastly, region III is situated at the carboxy-terminus of human RNase H1 and contains an amino acid sequence that is highly conserved with the amino acid sequence of *E. coli* RNase H1. Included within region III are the conserved amino acid residues that form the putative catalytic site, the second divalent cation binding site, and the basic substrate-binding domain of the *E. coli* enzyme.

Catalytic Triad

The three amino acids (Asp-10, Glu-48 and Asp-70) that make up the catalytic site of *E. coli* RNase H1 were identified by site-directed mutagenesis (20). These amino acid residues have also been shown to be involved with the coordination of the requisite divalent cation cofactor, (25). Comparison of the amino acid sequence of *E. coli* RNase H1with the amino acid sequences of the RNase H domain of retroviruses and RNase H1 from yeast, chicken, Human and mouse indicates that these three amino acid residues are conserved among all type 1 sequences (11).

Mutant proteins of human RNase H1 were prepared in which each of the three conserved catalytic residues Asp-145, Glu-186 and Asp-210 was substituted with, respectively, Asn, Gln and Asn. The complete ablation of cleavage activity observed for the RNase H1[D145N],

[E186Q] and [D210N] mutants indicates that all three of the conserved residues in human RNase H1 are required for catalytic activity (Table 1). The fact that the RNase H1[D145N] mutant competitively inhibited the activity of human RNase H1 suggests that the loss in cleavage activity observed for this mutant protein was not due to a loss in the binding affinity for the heteroduplex substrate (Fig. 4). Taken together these data suggest that, consistent with the *E. coli* enzyme, the three conserved residues likely form the catalytic site of the enzyme and are not involved in the substrate-binding interaction.

Basic substrate binding domain

The amino acid sequence of the basic substrate-binding region of *E. coli* RNase H1 is highly conserved in the human enzyme (11). The basic substrate-binding domain of *E. coli* RNase H1 has been extensively characterized and has been shown to comprise the α-helix III and following loop region of the enzyme (18, 20-21). The crystal structure of *E. coli* RNase H1 indicates that this region forms a relatively independent sub-domain with the loop region composed of a cluster of basic amino acid residues. These basic amino acid residues are believed to bind electrostatically to the phosphate backbone of the heteroduplex substrate.

Mutant proteins of human RNase H1 were prepared to determine whether these conserved basic amino acids served a similar function in the human enzyme. The RNase H1[K226,227A] mutant, in which two lysine residues were substituted with alanine residues, exhibited a cleavage rate two orders of magintude slower than the wild-type human enzyme (Table 1). The alanine substitution of all four lysine residues within the putative substrate-binding domain of human RNase H1 (RNase H1[K226,227,231,236A]) resulted in the complete loss of RNase H activity. Furthermore, the RNase H1[K226,227,231,236A] mutant was shown to competitively inhibit the cleavage activity of wild-type human enzyme (Fig. 4), suggesting that the observed loss of RNase H activity for the mutant protein was not due to a loss in the overall binding affinity of the mutant protein for the substrate.

The properties observed for basic amino acid residues of human RNase H1differ significantly from those observed for the *E. coli* enzyme. First, unlike the human enzyme the alanine substitution of any two of the basic residues within the substrate-binding domain of *E. coli* RNase H1did not affect the cleavage activity of the mutant *E. coli* proteins, i.e., the cleavage rates for the alanine substituted mutants were comparable to the rates observed

for the wild-type *E. coli* enzyme (18). Second, alanine substitution of four basic amino acids within the substrate-binding domain of *E. coli* RNase H1 resulted in a 5 - 10-fold reduction but not the ablation of the RNase H activity of the *E. coli* mutants. In addition, a 30 to 60-fold increase in the K_m was observed for these mutant proteins suggesting that a reduction in the binding affinity for the substrate was responsible for the observed reduction in cleavage activity. In the case of human RNase H1 mutant, the complete loss in cleavage activity for the RNase H1[K226,227,231,236A] mutant did not coincide with a loss in binding affinity for the substrate.

Clearly, these conserved basic residues appear to perform different functions within the two RNase H1 enzymes. Whether these substituents are interacting directly with the substrate is not clear, but the fact that the alanine substitution of the conserved lysine residues resulted in the reduction and/or ablation of the RNase H activity indicates that these residues are essential for the catalytic processes of the human enzyme. In contrast to the human enzyme, the basic residues of the *E. coli* enzyme appear to be the key substituents that make up the binding surface of the protein. Furthermore,

alanine-scanning mutagenesis of the basic residues of the substrate-binding domain of *E. coli* RNase H1 suggested that the binding interaction has more to do with the net positive charge of the basic amino acid cluster than on specific interactions involving specific basic amino acid residues of the *E. coli* enzyme (18). Finally, our data also suggest that other regions within human RNase H1 are contributing to the binding affinity of the enzyme. One likely region that may be involved in substrate binding is the dsRNA-binding domain of the human enzyme.

Region I

Human RNase H1 has been observed to contain the canonical α-β-β-β-α structure consistent with the dsRNA-binding motif (11). The position of the double-strand RNA binding domain at the amino-terminus of the enzyme is consistent with the structure observed for RNase H1 from *Saccharomyces cerevisiae* (26). The human RNase H1 dsRNA-binding domain differs from the yeast enzyme in that the human sequence appeared to correspond to a more complete dsRNA-binding motif. The properties of the dsRNA-binding domain of human RNase H1 were also observed to differ from the yeast enzyme in that the dsRNA-binding domain of the human enzyme was not modulated by divalent cation concentration. For example, RNase H1 from *Saccharomyces*

cerevisiae was observed to bind to dsRNA at Mg^{2+} concentrations below those required to activate the enzyme and was inhibited from binding to dsRNA at Mg^{2+} concentrations that activated the enzyme. In other words, binding to dsRNA and the RNase H activity of the*Saccharomyces cerevisiae* enzyme were observed to be mutually exclusive. Contrary to the yeast enzyme, the binding of human RNase H1 to dsRNA was determined not to be affected by Mg^{2+} concentrations required to activate the enzyme.

Deletion mutants of human RNase H1 were prepared in order to investigate the role of the dsRNA-binding domain, i.e. region I. These mutants included the deletion of the dsRNA-binding domain (RNase H1[ΔI]) and deletion of both the dsRNA-binding domain and region II between the dsRNA-binding domain and the conserved *E. coli* RNase H1 region (RNase H1[ΔI-II]). Both mutants in which the dsRNA-binding domain was deleted cleaved the heteroduplex substrate at a rate comparable to the rate observed for the wild-type enzyme (Table I). A mutant of RNase H1 from *Saccharomyces cerevisiae* in which the dsRNA-binding domain was deleted also exhibited RNase H activity (26). In light of the fact that the RNase H activity and dsRNA-binding properties of the yeast enzyme are mutually exclusive, it is not

surprising that the yeast enzyme would remain active with the dsRNA-binding domain deleted. On the other hand, the robust cleavage activity of the human deletion mutants is surprising, particularly when considering that the RNase H1[K226,227,231,236A] mutant was able to bind to the heteroduplex substrate. The cleavage activity of the RNase H1[ΔI] and [ΔI-II] mutants suggests that the enzyme does not require the dsRNA-binding domain in order to bind to the heteroduplex substrate. In fact, the binding affinity of the wild-type human enzyme for the heteroduplex substrate was <2-fold tighter than the RNase H1[ΔI-II] mutant without the dsRNA-binding domain (Table II). Clearly both the basic substrate-binding domain and the dsRNA-binding domain contribute to the overall binding affinity of human RNase H1 as the elimination of either domain did not affect the ability of the enzyme to bind to the substrate. It is important to note that these human RNase H1 proteins are His-tag fusion proteins and it is possible that the binding properties of the proteins may be enhanced by the His-tag. Numerous studies have shown that a His-tag does not interfere with nucleic acid binding properties as it is very small (few amino acids), and its pK is near neutral (27-28).

The cleavage pattern for the mutants in which the dsRNA-binding region was deleted (RNase H1[I] and [I-II]) differed from the pattern observed for the wild-type human enzyme. In fact the cleavage pattern for the RNase H1[I] and [I-II] mutants resembled the cleavage pattern of the *E. coli* RNase H1 enzyme which does not contain a dsRNA-binding domain. Taken together these data suggest that the dsRNA-binding domain is responsible for the observed strong positional preference for cleavage exhibited by human RNase H1, (16) and further suggest that this region contributes to the overall binding affinity of the enzyme for the substrate and the regulation of the sites of cleavage. Finally, the broad cleavage pattern observed for the RNase H1[I-II] mutant further suggests that the strong positional preference for cleavage displayed by human RNase H1 is not responsible for slower cleavage rate of the human enzyme compared to *E. coli* RNase H1. The cleavage rate observed for human RNase H1 was approximately two orders of magnitude slower than the rate observed for the *E. coli* enzyme (17). The strong positional preference for cleavage displayed by human RNase H1 in effect limits the number of productive binding interactions for a given substrate. Considering that RNase H1[I-II] mutant displayed a similar cleavage pattern to

the *E. coli* enzyme, the slower cleavage rate observed for human RNase H1 is likely not due to the strong positional preference for cleavage.

The role of the dsRNA-binding domain is not clear. Obviously the dsRNA-binding domain of human RNase H1 is not required for RNase H activity and consequently this region likely serves another function. RNase H1 enzymes have been proposed to participate in DNA replication and are believed to aid in the removal of the RNA primers during the DNA replication of the lagging strand. The strong positional preference for cleavage exhibited by the human RNase H1 proteins containing the dsRNA-binding domain is consistent with the average length of the RNA primers which have been shown to range from 7 - 14 nucleotides (29). Therefore the role of the dsRNA-binding domain of human RNase H1 may be to place the enzyme in the appropriate position on the RNA primer in order to ensure efficient removal of the primer.

Region II

Region II comprises the amino acid sequence between the dsRNA-binding domain (region I) and the conserved *E. coli* RNase H1 domain (region III). Deletion of this region (RNase H1[II]) resulted in a significant loss in the cleavage activity when compared to the wild-type enzyme.

The RNase H1[II] mutant was also shown to competitively inhibit the cleavage activity of human RNase H1 suggesting that the loss in RNase H activity did not appear to be due to a reduction in the binding affinity of the RNase H1[II] mutant for the heteroduplex substrate. Consequently, one possibility for the loss in RNase H activity observed for the RNase H1[ΔII] mutant may be due to the misfolding of the enzyme into an inactive conformation as a result of deleting region II. Alternatively, deletion of region II places the dsRNA-binding domain immediately adjacent to the conserved *E. coli* RNase H1 region of the human enzyme which may result in steric hindrance of the catalytic site by the dsRNA-binding domain.

The loss of cleavage activity observed for the RNase H1[II] mutant is consistent with that observed for RNase H1 of *Trypanosoma brucei* in which the deletion of this region also resulted in the loss of enzymatic activity (A. Campbell, personal communication). Furthermore, this region in the *T. brucei* enzyme has been shown to contain numerous acidic residues and site-directed mutagenesis of the acidic amino acids within this region also resulted in the ablation of the enzymatic activity. The highly acidic nature of this region is consistent in the human enzyme

and is found in RNase H1 proteins of *Crithidia fasciculata*, *Drosophila melanogaster* and *Saccharomyces cervisiae*. It is unclear how these acidic residues contribute to the enzymatic activity of human RNase H1, but this region appears to play a critical role in the structure of the enzyme. Clearly, understanding the role of this region with respect to the enzymatic activity of human RNase H1 warrants further investigation.

Region III

Region III, as represented by the H1[I-II] mutant, contains the conserved *E. coli* RNase H1 domain. The cleavage rate observed for the H1[I-II] mutant was comparable to the rate observed for wild-type human enzyme (Table I), but approximately two-orders of magnitude slower than the cleavage rate observed for *E. coli* RNase H1 (17). The robust activity of the RNase H1[I-II] mutant indicates that region III is capable of folding into an active structure independent of regions I and II and further suggests that region III constitutes an autonomous sub-domain of the human enzyme. Folding of the *E. coli* RNase H1 enzyme has been shown to follow a two-step process involving a core folding intermediate (28). The amino acid sequence of the core folding intermediate is highly conserved in the human enzyme, which shares 41%

amino acid identity with the *E. coli* enzyme, and suggests a similar folding pathway for human RNase H1[I-II] mutant. Whether the wild-type human enzyme follows a similar folding pathway remains to be determined.

The binding affinity of the RNase H1[I-II] mutant for the heteroduplex substrate was determined to be approximately 10-fold tighter than the binding affinity observed for the *E. coli* enzyme (Table II). Furthermore, the binding affinity of the wild-type human enzyme was <2-fold tighter than that observed for the RNase H1[I-II] mutant. Taken together, these data suggest that region III is providing a significant contribution to the increased binding affinity observed for human RNase H1. The tighter binding affinity observed for both the wild-type and human RNase H1[I-II] mutant likely limits the turnover rate of the human enzyme and may account for the two orders of magnitude slower cleavage rate of the human proteins compared to *E. coli* RNase H1 (17).

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